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TECHNOLOGY REPORT

Removal of the Floxed neo Gene from a Conditional Knockout Allele by the Adenoviral Cre Recombinase in vivo

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Summary: Conditional and tissue specific gene targeting using the Cre-loxP recombination system in combination with established ES cell techniques has become a standard for in vivo loss of function studies. In a typical flox and delete gene targeting strategy, the loxP-neo-loxP cassette is inserted into an intron and an additional loxP site is located in one of the homology arms so that loxP sites surround a functionally essential part of the gene. The neo cassette in usually removed by transient expression of the Cre recombinase in ES cells to avoid selection gene interference and genetic ambiquity. However, this causes a significant increase in manipulation of ES cells and often compromises ES cell pluripotency. Here we describe a method in which the floxed neo gene is removed from a knockout allele by infecting 16-cell-stage morulae by the recombinant Cre adenovirus. This virus provides only transient Cre expression and does not integrate into the mouse genome. Produced mosaic mice transmitted the desired allele without the neo cassette with high frequency to their offspring. This method is rapid and easy and does not require any special equipment. Moreover, because superovulated mice can be used as donors, this method does not necessitate a large number of mice. genesis 31:126–129, 2001.

Key words: Adeno Cre; neo removal; conditional knockout

INTRODUCTION

During the past decade, gene targeting through homologous recombination in ES cells has become a widely used technique to study gene function in vivo (Capecchi, 1989). However, total inactivation of gene function (gene knockout) often leads to embryonic lethality and thus limits the usefulness of the method. This problem can be overcome by using conditional gene-targeting methods, such as the Cre-loxP recombination system of bacteriophage P1, which has been shown to be an irreplaceable tool for conditional genome alterations (Rajewsky *et al.*, 1996; Nagy, 2000).

Today, the most common Cre/loxP strategy involves utilization of three loxP sites (Torres and Kuhn, 1997). Two of these sites flank a selectable marker, which is usually the neo cassette inserted into an intron, and the third one is located in one of the homology arms so that the loxP sites flank a functionally fundamental part of the gene. This design allows removal of a selection marker cassette, which is important because its presence in intron sequences has often been shown to dramatically reduce expression levels (Nagy *et al.*, 1998). Removal of a selection marker is commonly achieved by transiently expressing the Cre recombinase in ES cells (Torres and Kuhn, 1977; Joyner, 2001). However, this approach includes an additional electroporation and extended culturing period, which often compromises pluripotency of ES cells. Moreover, it has been reported that this step is not an easy one and that success is often gene dependent (Nagy, 2000). Therefore, alternative methods to remove the loxP-neo-loxP cassettes are of significant importance. Here we report a method based on infection of morulae with replication deficient recombinant adenoviruses expressing the Cre recombinase. This method is quick, easy, and efficient. In addition to removal of the floxed neo gene from the targeted locus, it can also be used to produce complete knockout alleles.

A targeting vector was generated by inserting the pgk-neo cassette flanked by the loxP sites into intron 7 and a single loxP site into intron 6 of the *alk2* gene (Fig. 1). This allows *alk2* inactivation by removal of exon 7 that encodes a part of the critical kinase domain. This construct was introduced into R1 ES cells using standard methods, and several clones that had undergone the desired homologous recombination event were obtained (targeting efficiency: seven out of 144 double-resistant

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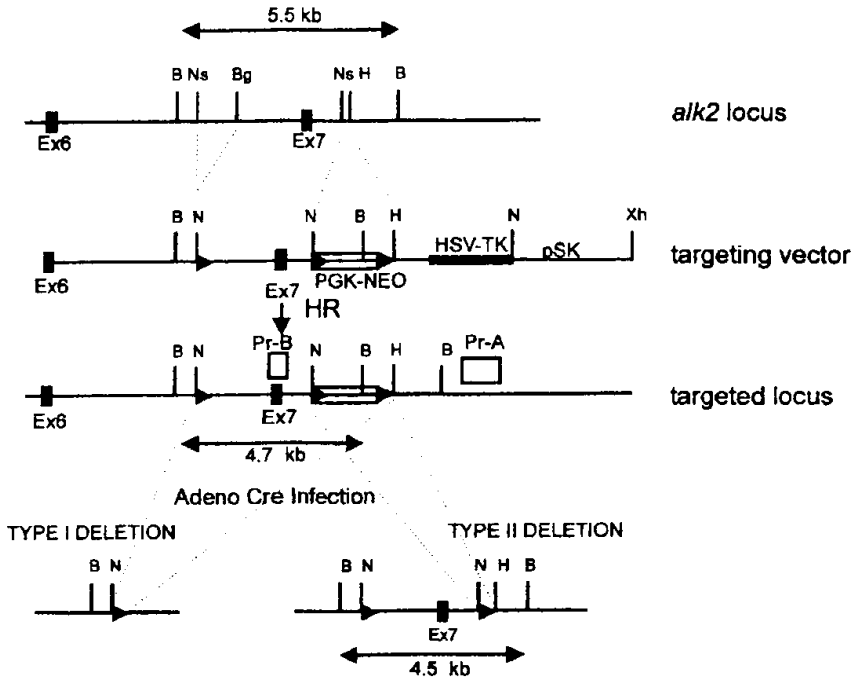

FIG. 1 A strategy to generate the conditional *alk2* allele. The *alk2* targeting vector was generated by replacing the *NsiI-BglII* fragment in intron 6 and the *NsiI-HindIII* fragment in intron 7 with the single loxP site and the loxP-pgkneo-loxP cassette, respectively. This vector was used to generate heterozygous mice, of which genotype was analyzed using *PstI* digestion and the external probe A (Pr-A) as well as *BamHI* digestion and the internal probe B (Pr-B). Predicted *BamHI* fragment sizes when probed with the exon 7-specific internal probe B (Pr-B) for wild-type allele, targeted allele, and type II deletion were 5.5 kb, 4.7 kb, and 4.5 kb, respectively. Type I deletion is not detectable with the probe B. Ns = *NsiI*; Bg = *BglII*; B = *BamHI*; H = *HindIII*; N = *NotI*; black triangle = loxP site; HR = homologous recombination.

Table 1
Summary of Site-Specific Recombinations in Tail DNAs of Mice Derived from AxCANCre Infected Morulae

	Number	Percentage (%)
Morulae infected	65	—
Blastocysts transplanted	46	—
Pups born	22	100
Neo present[a] (no recombination)	7	32
Neo lost[a] (recombination)	15	68
Exon 7 present (type II deletion)	4	18 (27)[b]
Exon 7 lost (type I deletion)	11	50 (73)[b]

[a]Tail DNAs were analyzed.
[b]Percentage of samples that had undergone recombination.

clones). Three randomly chosen cell lines were injected into blastocysts. Several of the obtained highly chimeric male mice were complete germ line transmitters. Homozygote males in an outbred background were without any obvious phenotypes. They were crossed with superovulated females and embryos (all of which were heterozygotes) were harvested at E1.5 (1.5 days postcoitum). The zona pellucida was removed and the embryos were infected with AxCANCre adenoviruses followed by thorough washing with B16 medium. When the infected embryos reached an early blastula stage they were transplanted back into uterine horns of 2.5 day pseudopregnant surrogate females.

Twenty-two pups were born (Table 1) and analysis of tail DNAs revealed that 15 samples had either totally lost neo or displayed a dramatic reduction in the neo signal (Fig. 2). When analyzed using the exon 7 specific probe, four samples essentially showed the desired hybridization pattern characteristic for the type II deletion, ie, excision of the loxP flanked neo gene. Subsequently, filters hybridized for neo were exposed for 4 days (standard exposure time is overnight) to see whether samples that seemed to have undergone the type II deletion would display any residual neo signal. Indeed, three of the four candidate samples were still weakly positive for neo, suggesting that these samples were mosaic, and displayed, at least in the tail tissue, recombinations other than the desired type II deletion, albeit with lower frequency. Two of the mosaic mice with the type II deletion transmitted the desired type II recombination to their offspring with high frequency (Fig. 2B). None of the samples showed a positive signal with the Cre-specific probe (data not shown). This indicates that AxCANCre leads only to transient Cre expression in infected cells.

In the present study we show that the adenoviral Cre recombinase can be used to introduce recombinations into the mouse genome containing loxP sites. Recently, Xu and colleagues described two methods for direct removal of a floxed allele: one based on crossings with the EIIa-Cre transgenic mice, another based on microinjection of the Cre expression construct into pronuclei of fertilized eggs (Xu *et al.*, 2001). The transgenic approach required two rounds of matings and produced the desired genotype with a relatively low frequency, ie, in the final round of matings only one out of 43 pups displayed an excision of neo. Similar to the transgenic approach, the microinjection approach produced only one mouse of the desired genotype out of 320 microinjections.

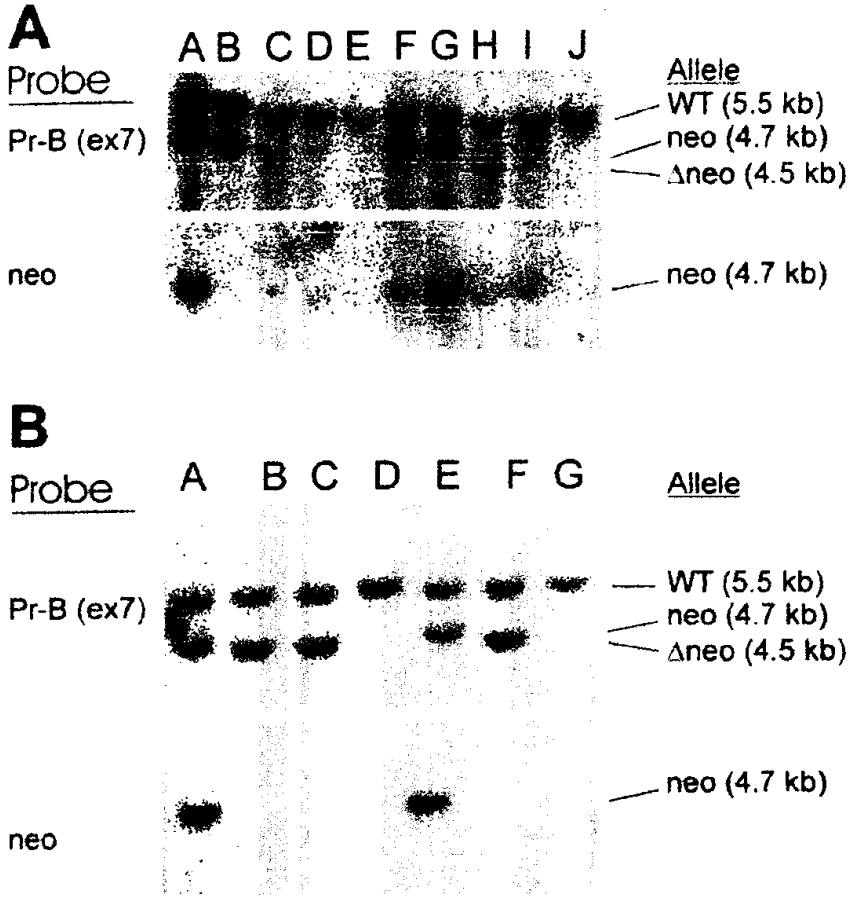

FIG. 2 (**A**) Adeno Cre-induced recombination of the targeted *alk2* locus. Tail DNAs were analyzed by Southern blotting using *Bam*HI digestion and hybridization with the exon 7-specific probe B (upper panel) and the neo-specific probe (lower panel). Samples A and G show no recombinations, while samples E and J display the complete type I deletion. B shows the complete type II recombination, whereas F and H are mosaic. They are composed of cells that show no recombinations (weak neo signal) and of cells that have undergone the type II deletion. C, D, and I are also mosaic; they are composed of cells that have undergone both type I and type II deletions, ie, no neo signal but uneven intensity between wt and mutated (Δneo) alleles. (**B**) Genotypes of selected offspring displaying different recombinations. Mosaic mice were crossed with B6xCBA (f1) mice and offspring were genotyped. Tail DNAs were analyzed using Southern blot analysis (*Bam*HI digestion) and hybridization with the exon 7 (upper panel) and neo (lower panel) specific probes. Samples A and E are heterozygotes for the targeted allele (neo present), samples B, C, and F display the type II deletion, sample D is a wild-type, and sample G displays a putative type I deletion (compared to the wt allele G displays 50% reduction in the hybridization signal intensity).

Moreover, this approach is tedious and requires both the equipment for and expertise in microinjection. Transgenic EIIa-Cre mice were also used by Holzenberger and colleagues to create floxed substrains (Holzenberger *et al.*, 2000). They described that only mosaic EIIa-Cre/loxP males could be used to create type II deletions, as mosaic females consistently generated the complete excision (type I deletion) during oogenesis.

MATERIALS AND METHODS

Construction of the *alk2* Targeting Vector and Generation of Mutant Mice

The mouse *alk2* locus was clone from the 129x1/SvJ genomic library (Stratagene). The *alk2* targeting vector was constructed by replacing a 200-bp *NsiI-HindIII* fragment in intron 7 with the loxP-pgkneo-loxP casette. An additional loxP site was introduced into intron 6 by replacing the 0.6-kb *NsiI-BglII* fragment with the loxP site. Negative selection was with the HSV-TK gene. The targeting vector was linearized with *XhoI* and electroporated into R1-ES cells (Kaartinen *et al.*, 1995; Voncken *et al.*, 1995). Double selection and clone expansion was carried out as described (Kaartinen *et al.*, 1995; Voncken *et al.*, 1995). ES cells were screened for the presence of the correctly targeted locus by Southern blot analysis using *PstI* digestion and hybridization with the external probe A (Fig. 1). Chimeric mice were generated by microinjection of the correctly targeted ES cell clones into blastocysts of C57BL/6 mice as described (Papaioannou and Johnson, 1993). Highly chimeric male mice were bred with B6xCBA (f1) females and offspring with the coat color characteristic for the ES R1 background (yellow belly) were tested for germ line transmission by Southern blot analysis as described above. Homozygous mice were obtained by interbreeding heterozygotes.

Infection of Morulae with AxCANCre Adenoviruses

Homozygote alk2 mutants were bred with superovulated B6xCBA (f1) females. At E1.5 (1.5 days postcoitum) 16-cell-stage morulae were isolated, the zona pellucida was removed by acidic Tyrode's solution as described (Hogan *et al.*, 1994), and embryos were infected with AxCANCre adenoviruses (from RIKEN, Japan; 1×10^7 viral particles per 1 ml of B16 medium) for 3 h followed

by thorough washing with B16 medium. When the embryos reached the early blastocyst stage they were transplanted back into uterine horns of 2.5 day pseudopregnant B6xCBA (f1) surrogate females as described (Hogan *et al.*, 1994). Genotypes of obtained offspring were analyzed by Southern blot analysis as described above. All the animal procedures were carried out according to the national and institutional quidelines.

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